MOLECULAR AND CELLULAR BIOLOGY, Dec. 2006, p. 8781–8790 Vol. 26, No. 23
0270-7306/06/$08.00+0 doi:10.1128/MCB.01409-06

Chromatin Structure and Transcription of the R1- and R2-Inserted rRNA Genes of *Drosophila melanogaster*▽

Junqiang Ye† and Thomas H. Eickbush*

University of Rochester, Department of Biology, Rochester, New York 14627

Received 1 August 2006/Accepted 1 September 2006

About half of the rRNA gene units (rDNA units) of *Drosophila melanogaster* are inserted by the retrotransposable elements R1 and R2. Because transcripts to R1 and R2 were difficult to detect on blots and electron microscopic observations of rRNA synthesis suggested that only uninserted rDNA units were transcribed, it has long been postulated that inserted rDNA units are in a repressed (inactive) chromatin structure. Studies described here suggest that inserted and uninserted units are equally accessible to DNase I and micrococcal nuclease and contain similar levels of histone H3 and H4 acetylation and H3K9 methylation. These studies have low sensitivity, because psoralen cross-linking suggested few (estimated <10%) of the rDNA units of any type are transcriptionally active. Nuclear run-on experiments revealed that R1-inserted and R2-inserted units are activated for transcription at about 1/5 and 1/10, respectively, the rate of uninserted units. Most transcription complexes of the inserted units terminate within the elements, thus explaining why previous molecular and electron microscopic methods indicated inserted units are seldom transcribed. The accumulating data suggest that all units within small regions of the rDNA loci are activated for transcription, with most control over R1 and R2 activity involving steps downstream of transcription initiation.

Initial cloning of the repeated rRNA genes (rDNA units) of *Drosophila melanogaster* revealed large insertions within the 28S rRNA gene (Fig. 1) (28, 63). These sequences, originally termed type I and type II insertions, were eventually identified as two distinct lineages of site-specific non-long-terminal-repeat retrotransposons and renamed R1 and R2 (33). Typically half (range, 32 to 77%) of the rDNA units in different geographical strains of *D. melanogaster* are inserted by R1 or R2 (34). Full-length R1 and R2 insertions are 5.3 kb and 3.6 kb, respectively, but many insertions are truncated to variable extents at their 5′ end. All elements are inserted in the same transcriptional orientation as the 28S gene. The remarkable specificity of each element is dependent upon the combined action of a specific endonuclease that cleaves the target site and a reverse transcriptase that uses this cleavage to prime reverse transcription (2, 12, 46).

The sequences of inserted and uninserted rDNA units are identical (38, 45), a reflection of the frequent retrotransposition of R1 or R2 elements into the rDNA units and their elimination by recombination (4, 34, 53, 54). In spite of this rapid turnover, phylogenetic analyses have revealed that these elements have been vertically maintained in insect lineages since the origin of arthropods (6, 23, 48). More recent studies have revealed R2 or related non-long-terminal-repeat retrotransposons inserted near the R1 and R2 sites in the rRNA genes of nematodes, platyhelminthes, tunicates, and vertebrates (7, 8, 21, 39).

Previous studies of inserted rDNA units have suggested that both the insertions and the rDNA units they inhabit are not transcribed. Northern blots of total RNA from various tissues of *D. melanogaster* have revealed only low levels of R1 and R2 transcripts derived from both the full-length and 5′ truncated elements (18, 37, 43). These low levels were similar to that derived from the cotranscription of foreign sequences inserted into the 28S gene target site (20). Direct electron microscopic observations of actively transcribing rDNA units revealed few RNA polymerase-nasent RNA complexes longer or shorter than expected for uninserted rDNA units (10, 11, 26, 35). These findings have given rise to the model that the inserted rDNA units are silenced at the chromatin level, with the low levels of transcripts occasionally observed derived from sporadic read-through transcription (18, 27, 43).

That a large fraction of the rDNA units in an organism could be sequestered from the transcriptional machinery is consistent with findings that there is considerable plasticity in the number of rDNA units in any organism (44). Most organisms appear have more than the minimum number of rDNA units needed for full viability. In eukaryotes as diverse as yeast, plants, and mammals, less than half of the units appear to be active at a given time (13–15). The active rDNA units are more accessible to nucleases and cross-linking reagents than the inactive units. While the establishment of these two rDNA chromatin states is not understood, epigenetic factors, such as histone modifications and in some organisms DNA methylation, are either the cause or the consequence of the different chromatin states (review in reference 29).

Previous studies of the chromatin structure of the rRNA genes of *D. melanogaster* either did not differentiate between the inserted and uninserted units (3, 40, 61) or indicated that the inserted units were less accessible (62). In this report, we compared the chromatin structures of inserted and uninserted rDNA units of *D. melanogaster* and conducted nuclear run-on experiments to directly measure their rates of transcription. We found the inserted and uninserted rDNA units of embry-

* Corresponding author. Mailing address: Department of Biology, University of Rochester, Rochester, NY 14627. Phone: (585) 275-7247. Fax: (585) 275-2070. E-mail: eick@mail.rochester.edu.

† Present address: Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138.

▽ Published ahead of print on 25 September 2006.

TABLE 1. Primers used in the ChIP experiments

Fragment	Primer sequences	GenBank accession no. (coordinates)
TOT	5′-GGAACGGGCTTGGAATAATTAGCG-3′ and 5′-GAAGCTTGCATCAAAACCCAATACC-3′	M21017 (6010–6259)
UN	5′-AAACAAAGCATTGTGATGGCCCTAG-3′ and 5′-CTAATTATTCCAAGCCCGTTCCCTT-3′	M21017 (5784–6031)
R1-5′	5′-CGGACGTGTTTTCGTTGCGCT-3′ and 5′-CCTTAGCGGTGACTACCACCAATAA-3′	X51968 (1–255)
R1-3′	5′-GCTGTGACCCAGAGATCAGTAGAGAT-3′ and 5′-GGATCCCTCCGAACTTATTTTACG-3′	X51968 (5090–5343)
R2-5′	5′-TTGGGGATCATGGGGTATTTGA-3′ and 5′-TGCTTGTAGTTCCAATATGAATAAATTTCC-3′	X51967 (1–250)
R2-3′	5′-TAGCTAAATCGTTTGGTTCAAAACA-3′ and 5′-TTTTGATCGCGGAGGTATGG-3′	X51967 (3352–3589)
ACT	5′-AGCGAGCAGAAGTCCAAAAG-3′ and 5′-GCGACCCTCAGTCGTTTTAG-3′	X12452 (147–307)
CEN	5′-CTGTCCCGTACTCGTCTCGT-3′ and 5′-GGACCCAATACGGTACCACT-3′	M86309 (229–461)

onic cells cannot be differentiated based on their accessibility to nucleases, psoralen cross-linking, and histone 3 and 4 modifications. The nuclear run-on experiments demonstrated that R1 and R2 elements are transcribed, but these transcripts unusually terminate within the element. These findings are discussed in relationship to the earlier studies as well as the possible mechanisms that regulate the activity of the R1 and R2 elements.

MATERIALS AND METHODS

Nuclear isolation and gene probes. Nuclei were isolated from ~0.5-g 1- to 16-h or 3- to 20-h embryos of the *D. melanogaster* line w^{1118} (64). After homogenization in nuclear buffer A (60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 15 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol [DTT], and 300 mM sucrose), debris was removed by centrifugation at 550 × *g* for 1 min, and the nuclei were pelleted by centrifugation at 1,300 × *g* for 10 min. Nuclei were resuspended in nuclear buffer A and further purified by sedimentation through a 1.7 M sucrose cushion (60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 15 mM Tris-HCl, pH 7.5, 0.5 mM DTT, and 1.7 M sucrose) at 28,000 × *g* for 20 min.

The probes used in this study were prepared by PCR amplification of cloned fragments or genomic DNA and are summarized in Fig. 1. The location of each fragment is as follows: for GenBank accession number M21017, 240 repeat (240), 10081 to 10323; promoter (Pro), 10636 to 10866; ETS, 10872 to 11105; 18S gene, 901 to 1140; ITS-1 (internal transcribed spacer [ITS]), 2202 to 2451 or 1953 to 2452; 28S gene (28S), 5784 to 6031; for GenBank accession number X51968,: R1 5′ end, 1 to 255 or 1 to 507; R1 3′ end, 5090 to 5343 or 4838 to 5343; for GenBank accession number X51967, R2 5′ end, 1 to 250 or 1 to 510; R2 3′ end, 3352 to 3589 or 3076 to 3589. The probe for Fig. 3 corresponded to sequence 6254 to 6554 of M21017, and the 18S/ITS probe in Fig. 5A was sequence 901 to 2451.

Nuclease digestions and Southern blotting. Nucleus pellets were resuspended in the nuclear digestion buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT), DNase I was added, and incubation was done at 24°C for the specified time. The reaction was stopped by adding EDTA and sodium dodecyl sulfate (SDS) to final concentrations of 10 mM and 0.02%, respectively. For micrococcal nuclease (MNase) digestion, the nucleus solutions were made 3 mM $CaCl_2$ and 0.5 units/μl MNase (Fermentas) and incubated at 24°C for the specified time. Reactions were stopped as in the DNase I digestions. After DNase I or MNase digestion, the DNAs were extracted from the nuclei and subjected to Southern blotting (33).

ChIP experiments. Chromatin immunoprecipitation (ChIP) assays (9) utilized 1 g of 1- to 16-h dechorionated w^{1118} embryos, which were cross-linked in 10 ml of 50 mM HEPES, 1 mM EDTA, pH 8.0, 100 mM NaCl, and 1.8% formaldehyde in the presence of 30 ml *n*-heptane for 15 min. Embryos were gently spun down and washed for 10 min, first with 50 ml of 0.125 M glycine and 0.01% Triton X-100 in phosphate-buffered saline, next with 10 ml of 10 mM HEPES, pH 7.6, 10 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, and 0.25% Triton X-100, and finally with 10 ml of 10 mM HEPES, pH 7.6, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 0.01% Triton X-100. Embryos were then resuspended in 5.5 ml of sonication buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 0.5 mM EGTA, pH 8.0) and homogenized with a type A pestle for three strokes in a 7-ml Dounce homogenizer. The homogenate was transferred to a 15-ml conical tube and sonicated for 30-s intervals four times with a Branson Sonifier 450 at setting 6 of constant power. After spinning at 4,000 × *g* for 30 min at 4°C, the supernatant was collected and adjusted to low-salt RIPA buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA, pH 8.0, and 1× complete protease inhibitor [Roche]). Roughly 70-μg aliquots of chromatin were separately incubated with 10 μl of anti-acetyl H3, anti-acetyl H4, and anti-trimethyl H3K9 antibodies (Upstate Biotechnology) at 4°C overnight. Antibody-chromatin immune complexes were recovered by adding N-protein A beads (Amersham Biosciences) preequilibrated with low-salt RIPA containing 100 μg/ml herring sperm DNA and further incubated for 2 h. Samples were centrifuged at 6,000 rpm for 30 s and the pellets washed five times with low-salt RIPA buffer, two times with high-salt RIPA buffer (as above but with 500 mM NaCl) with 100 μg/ml herring sperm DNA, and once with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0 and finally resuspended in 100 μl of the same buffer. RNase A was added to a final concentration of 100 μg/ml and incubated at 37°C for 30 min. Proteinase K and SDS were then added to final concentrations of 0.5 mg/ml and 0.5%, respectively, and incubated at 65°C overnight to reverse the cross-linking. Samples were extracted with phenol-chloroform, chloroform, precipitated with ethanol in the presence of 20 μg glycogen, and subjected to PCR analysis. PCR cycles were adjusted to allow the amplifications to remain in the linear range. Primer sequences and coordinates for the amplified fragments are shown in Table 1.

Psoralen cross-linking. Psoralen cross-linking was conducted following the procedures of Sogo and colleagues (15, 47). A UVP model B 100-A UV lamp was used to irradiate nuclei from a distance of 6 cm for 5 min on ice in the presence of 10 μg/ml psoralen (4,5′,8-trimethylpsoralen; Sigma). The irradiations were continued for a total of 25 min, replenishing the psoralen at 5-min intervals. After DNA extraction and appropriate restriction enzyme digestion, the DNA samples were electrophoresed on 0.7% agarose gels. Before blotting to nitrocellulose membranes, gels were irradiated with short-wave UV (252 nm) for 2 h in the cold to reverse the cross-linking. Psoralen cross-linking of *Saccharomyces cerevisiae* strain Y728 (5) was conducted under the same conditions and using the same restriction enzymes and probe as previously described (15).

Nuclear run-on transcription assays. Nuclear run-on assays (31) were conducted with isolated nuclei in 0.3 ml of 90 mM KCl, 25 mM DTT, 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM (each) ATP, CTP, and GTP, and 150 μCi [α-^{32}P]UTP (3,000 Ci/mmol) with or without 100 μg/ml α-amanitin (Sigma). Incubations were at 25°C or 37°C for 15 min, the reactions stopped by the addition of 30 mM EDTA and 2% SDS, and the RNA isolated. RNA from each nuclear run-on reaction was boiled for 5 min and hybridized to 0.5 μg denatured DNA fragments from various regions of the rDNA units bound to nitrocellulose paper (31). Hybridizations were conducted at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's, and 0.5% SDS for 20 h. Membranes were washed twice each in 2× SSC–0.1% SDS and 0.5× SSC–0.1% SDS at 65°C before exposure to a PhosphorImager screen. By using only regions of the rDNA that are processed from the major transcript, it was not necessary to eliminate by RNase treatment the 18S and 28S rRNA present in the nuclei (60).

To determine the relative hybridization efficiency, DNA fragments used in the nuclear run-on experiments were cloned to pCR-Blunt vector (Invitrogen) and sequenced. Clones containing the appropriate insert orientation were picked and digested with a restriction enzyme that cleaved the downstream end of the insertion. From these DNA templates, RNA probes were made by in vitro transcription with T7 RNA polymerase in the presence of[α-^{32}P]UTP and purified from a denaturing polyacrylamide gel. Equal counts of these RNA probes were pooled, hybridized to nitrocellulose membranes, and immobilized with identical amounts of DNA fragments under the same hybridization conditions used in the nuclear run-on experiments, and the relative hybridization efficiencies of the different DNA fragments was determined.

RESULTS

All regions of the rDNA units are packaged into nucleosomal arrays. Previous chromatin studies of the *D. melanogaster* rDNA units suggested that the promoter region was more accessible than the coding regions (40, 61). As a first step to compare the chromatin structures of the inserted and uninserted rDNA units, we monitored their MNase digestion patterns. Nuclei were isolated from 1- to 16-h embryos, a period of rapid activation and rRNA transcription (50). Nuclei were digested with MNase for increasing lengths of time, and the purified DNA was blotted and probed with short DNA fragments from different regions of the rDNA units. The locations of these probes and the percentages of the total rDNA units represented by the uninserted and various insertion classes are shown in Fig. 1. Because inserted and uninserted rDNA units are identical in nonelement sequences, the promoter region and most gene region probes hybridized equally well to inserted and uninserted units. On the other hand, the 28S probe was selected to span the R1 and R2 insertion sites. Most R1 insertions result in a 23-bp deletion of the target site upstream of the insertion (33), while most R2 insertions result in variable deletions of up to 40 bp (24). Because the 28S probe was both split and partially deleted by R1 and R2 insertions, this probe provided the best representation of the uninserted rDNA units.

Micrococcal digestion of embryonic nuclei for different times (Fig. 2A) revealed that all regions of the rDNA units were packaged into nucleosomal arrays similar to that of bulk DNA (stained DNA in panel at left). Scans of the longest nuclease digestion times from each blot are shown in Fig. 2B, while the fractions of the DNA digested to mono-, di- and trinucleosomes at 1 and 4 min are shown in Fig. 2C and D. The nucleosomal profile and the extent of digestion were similar for the 18S, 28S, R1, and R2 probes, suggesting that most inserted and uninserted units were packaged into nucleosomal arrays with similar accessibility to MNase.

Consistent with results of previous studies (40, 61), the 240-bp intergenic spacer repeats, the promoter, and the external transcribed spacer (ETS) digested more rapidly and gave a less-distinct nucleosome profile than the coding regions. Because these regions of rDNA are AT rich (69 to 73%; Fig. 2B) and MNase has been shown to have AT sequence preference (19, 56), it was possible that the faster digestion of the 5′ end of the rDNA units was a result of the AT richness of these regions. Data consistent with this explanation were obtained by MNase digestion of purified (protein-free) genomic DNA probed with the same eight segments of the rDNA unit (lane 1 in all panels of Fig. 2A). Free DNA corresponding to the area of the promoter digested more rapidly than both bulk DNA and the DNA corresponding to the 18S and 28S genes and R1 and R2 insertions. Further support for AT content affecting the rate of digestion of the different regions of the rDNA unit was obtained from the ITS probe. Located between the 18S and 28S genes, this sequence would be expected to have a chromatin structure similar to that of the genes. However, consistent with its higher AT content (Fig. 2B), the digestion rate of the ITS region was intermediate between the rate of cleavage of the 18S/28S genes and that of the promoter region in both isolated nuclei and free DNA. These data suggest that the majority of the inserted and uninserted units are assembled into nucleosomal arrays similar to that of bulk chromatin.

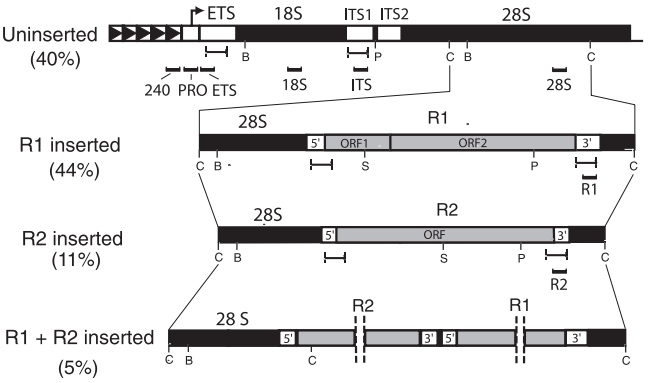

FIG. 1. Diagram of inserted and uninserted rDNA units of *D. melanogaster*. Each rDNA unit contains an 18S, 5.8S, and 28S rRNA gene (black boxes), an ETS, and two ITS regions (open boxes). Transcription starts at the arrow and terminates near the 3′ end of the 28S gene. The promoter region and 240-bp repeats located upstream of the promoter in the intergenic region between units are also diagramed. The R2 insertion site is 60 bp upstream of the R1 site (33). The open reading frames of the R1 and R2 elements are shown as gray boxes, while the 5′ and 3′ untranslated regions are shown as boxes labeled 5′ and 3′. The probes used in Fig. 2 are shown as thick bars below each diagram. The 0.5-kb DNA fragments used for the nuclear run-on assays in Fig. 6 are shown by the thinner bars below the gene diagrams. Also shown are the cleavage sites for the restriction enzymes BglII, B; ClaI, C; PstI, P; and SalI, S. The fraction of the rDNA units corresponding to each insertion type in the strain used for these studies is indicated in parentheses.

Inserted and uninserted units are equally accessible to DNase I. ClaI cleaves the 28S gene at sites flanking the R1 and R2 insertion sites but not within either element, while SalI and PstI cleave short distances from the 3′ end of each element (Fig. 3A). Using this ability to place inserted and uninserted rDNA units on different-size restriction fragments, the relative nuclease accessibilities of the different types of rDNA units could be compared. DNA extracted from DNase I-treated embryonic nuclei was followed by two sets of restriction digests: (i) ClaI plus SalI, which placed the DNA from R1- and R2-inserted units on larger fragments than those for the uninserted units, and (ii) ClaI plus PstI, which placed the DNA from the inserted units on shorter fragments than those for the uninserted units. After digestion the DNA was blotted and probed with a downstream region of the 28S gene (Fig. 3A). Because R1 elements are inserted downstream of R2 elements, rDNA units with both R1 and R2 insertions appeared as R1-inserted fragments in these blots.

The DNase I sensitivities of the uninserted, R1-, and R2-inserted units are shown in Fig. 3B. To control for variations in the amount of DNA loaded in each lane, a graph of the fraction of the three different rDNA fragments at each time point normalized to the fraction at time zero is shown in Fig. 3C. The restriction fragment derived from the R1-inserted units was most rapidly digested in the left panel, while the fragment from the uninserted units was most rapidly digested in the right panel. Thus, longer DNA fragments were more rapidly digested irrespective of whether they corresponded to inserted

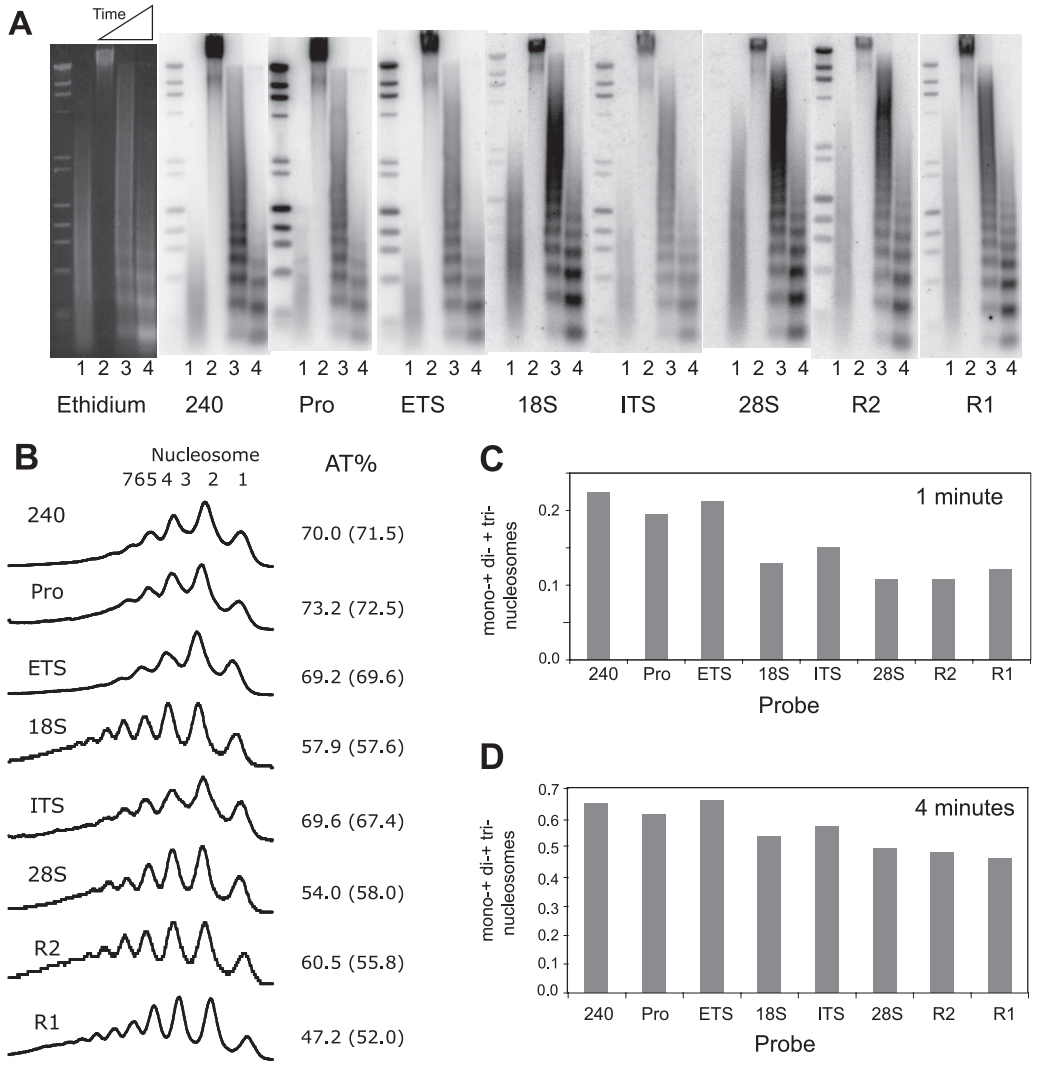

FIG. 2. Digestion of different regions of the rDNA units with MNase. (A) Isolated 1- to 16-h embryonic nuclei were digested with MNase (0.5 U/μl) at 24°C for 0, 1, or 4 min (lanes 2 to 4 of each panel). Purified (protein-free) genomic DNA was also digested with MNase (0.025 U/μl) for 30 s (lane 1 of each panel). The DNA from each digest was extracted, divided into eight aliquots, blotted, and hybridized with the probes diagramed in Fig. 1: the 240-bp intergenic spacer repeats (240), promoter (Pro), ETS, 18S, ITS1, 28S, R1, and R2. (B) Tracings of the signals from lane 4 of each panel in A. Listed at the right of each tracing is the AT content of each probe with the AT content of the flanking 2 kb listed in parenthesis. (C) Combined fraction of the total hybridization signal in the mono-, di-, and tri nucleosome fractions in the 1-min digestion (lane 3) of each panel in A. The combined signal in the mono-, di-, and trinucleosome fractions was divided by the total signal in the lane. (D) As in panel C but the fraction of the total signal in the mono-, di-, and tri nucleosome fractions in the 4-min digestions (lane 4).

or uninserted units. Only in the case of the ClaI-SalI digest were the R2 units somewhat more slowly digested than the uninserted units, even though the R2 units were on slightly larger fragments. These experiments again suggest that most inserted rDNA units are packaged into a chromatin structure similar to that of the uninserted units. While a previous report has suggested that uninserted units are more accessible to DNase I digestion than the inserted units in *D. melanogaster*, that report did not control for the size of the DNA restriction fragments being monitored (62).

Histone modifications of R1/R2 inserted and uninserted rDNA units. It has been shown that "active" genes are generally packaged with histone H3 and H4 containing hyperacetylated N-terminal tails (AcH3 and AcH4), while "silenced" genes are packaged with histone H3 hypermethylated at lysine 9 (MeH3K9) (36). The chromatin structures of active and inactive rDNA units have also been proposed to contain these differences in histone modifications (3, 52, 59). We therefore carried out ChIP experiments with antibodies to acetyl H3, acetyl H4, and trimethyl H3K9 to test whether the chromatin of R1- and R2-inserted units differed from the chromatin of uninserted units.

The ChIP assays were conducted with chromatin from 1- to 16-h embryos (9). As shown in Fig. 4A, a region of the 28S gene downstream of the R1 and R2 insertion sites was selected for amplification to represent all rDNA units (TOT, for total); the insertion site region of the 28S gene was selected to represent the uninserted units (UN); and the R1- and R2-inserted

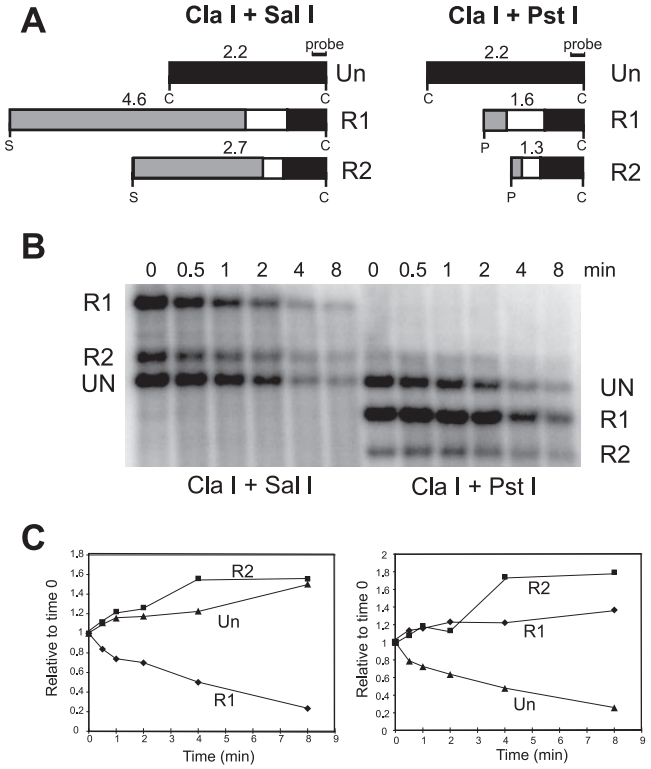

FIG. 3. Comparison of the DNase I sensitivity of the inserted and uninserted rDNA units. (A) Diagram of the DNA fragments generated by the restriction enzymes used in this experiment. ClaI sites (labeled C) are located 0.7 kb downstream and 1.5 kb upstream of the R1 insertion site in all rDNA units. The probe was a 300-bp fragment immediately adjacent to the downstream site. R1 and R2 elements do not contain ClaI sites but do have SalI and PstI sites at the positions shown (labeled S and P). (B) Isolated nuclei were digested with DNase I (0.05 U/μl) at 24°C for increasing times (0.5, 1, 2, 4, or 8 min). The DNA was purified and digested with ClaI plus SalI or ClaI plus PstI and subjected to Southern analysis. (C) Quantitation of the relative DNase I sensitivities of the inserted and uninserted rDNA units. For each digestion time, the proportions of the three different types of rDNA were normalized to the relative amount of that fraction at time zero.

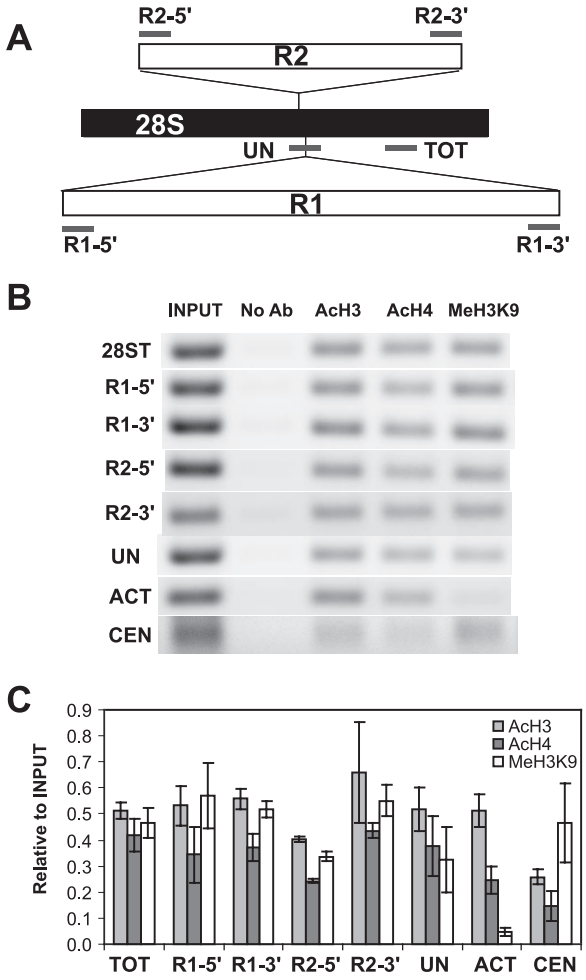

FIG. 4. The R1-inserted, R2-inserted, and uninserted rDNA units are packaged into chromatin structures that have similarly modified histone H3 and H4. (A) Diagram of the 28S gene and its insertions with the locations of the regions amplified from the 28S, R1, and R2 sequences. See Materials and Methods for more details, as well as the regions amplified from the actin gene and centromeric sequences. (B) PCR amplifications from the ChIP experiments. Each primer pair amplified five samples: (i) input DNA (1/3 of the original starting material), (ii) a control mock immunoprecipitation with no antibody, (iii) immunoprecipitation with anti-acetyl H3, (iv) immunoprecipitation with anti-acetyl H4, and (v) immunoprecipitation with anti-trimethyl H3K9. The PCR amplifications were empirically adjusted to be in the linear range: 25 cycles, the 28S genes, R1 elements, and centromeric satellite DNA; 27 cycles, R2 elements and the actin 87E gene; 30 cycles. Each centromeric DNA band shown represents the major band generated in a series of bands. (C) Relative enrichment of the amplification from each ChIP sample over that of the input sample. The graph represents the mean for three independent PCRs with the standard deviation shown.

units were monitored by separate amplification of regions near their 5′ and 3′ ends (R1-5′, etc.). All PCR amplifications were adjusted to be within the linear range and conducted in triplicate. Figure 4B shows the PCR products from one amplification, while the intensities of bands from all experiments were normalized to that of the input band and plotted in Fig. 4C. Confirmation of the approach and the specificity of the three antibodies was demonstrated by the enrichment of AcH3 and AcH4 and the absence of MeH3K9 in the chromatin of the transcriptionally active actin 87E gene (ACT) and a deficit of AcH3 and AcH4 and enrichment of MeH3K9 in the chromatin of a transcriptionally inactive centromeric sequence (CEN) (1).

AcH3 and AcH4 levels in the chromatin of the uninserted rDNA units and the R1 and R2 elements were similar to that of the actin gene, uniformly higher than that of the centromeric sequences, suggesting transcriptional activity. However, the levels of MeH3K9 in the chromatin of both the inserted and uninserted rDNA units were higher than that of the actin gene, more similar to that of the centromeric sequences, suggesting transcriptional inactivity. The presence of both H3/H4 acetylation and H3K9 methylation in the chromatin associated with the rDNA units is consistent with the model that only a fraction of these units need to be transcribed in any cell. A somewhat higher percentage of the uninserted units may be transcribed than of the inserted units, because the uninserted unit signal (UN) was the only one with a lower levels of the MeH3K9 modification than both H3 and H4 acetylation.

Psoralen cross-linking of inserted and uninserted rDNA units. Sogo and colleagues have demonstrated that transcriptionally active rDNA units are more accessible to psoralen and after UV cross-linking can be separated from the inactive units by their slower migration during electrophoresis (14). This psoralen accessibility has been used to detect active rDNA units in many organisms (13, 15, 16, 47). Isolated nuclei from 1- 16-h embryos were incubated with psoralen and UV cross-linked (15, 47, 58). To monitor all rDNA units, the cross-linked DNA was digested with BglII or BglII plus PstI and probed with an 18S/ITS-1 sequence (Fig. 5A, left panel). To separately score uninserted, R1-inserted, and R2-inserted rDNA units (right panel), the cross-linked DNA was digested with ClaI plus PstI and probed with the downstream 28S gene probe (the same digests and probe used for Fig. 3). As a control, psoralen cross-linking of the rDNA units from *Saccharomyces cerevisiae* was also conducted. After cross-linking, the *S. cerevisiae* DNA was digested with EcoRI and probed with a gene region spanning parts of the 18S and 25S genes (Fig. 5B).

Consistent with previous findings for *S. cerevisiae* (15, 16, 58), cross-linked DNA (lane X) migrated as two distinct bands slower than those for uncross-linked DNA (lane C). The more accessible (i.e., active) band corresponded to one-third of the total units (31% for the 18S band and 34% for the 25S band). In contrast to the yeast rDNA units, the cross-linked *D. melanogaster* rDNA units showed a single band that was slower migrating than that for the uncross-linked fragment. The shift in migration of the cross-linked *D. melanogaster* units was similar to that of the inactive units in yeast. This single cross-linked product was observed for the fragments in the left panel, representing all rDNA units, and for the fragments in the right panel, representing the individual uninserted or R1- or R2-inserted fractions. The series of faint lower and higher molecular bands seen in both the cross-linked and uncross-linked DNA corresponded to the greater restriction polymorphisms present in the rDNA units of *D. melanogaster*. Because of these polymorphisms, the lower limit in our ability to detect a second more slowly migrating band was about 10%. Assuming that actively transcribed rDNA units in *D. melanogaster* embryos are similar in structure to those of other organisms and thus would have been detected by psoralen cross-linking, these data suggest only a minor percentage of the inserted and uninserted units are actively transcribed. Similar results have been obtained with nuclei isolated from embryos of different ages and with different levels of cross-linking (data not shown).

Nuclear run-on transcription assays. As the most direct assay to monitor the fraction of the inserted and uninserted rDNA units that are actively being transcribed, we conducted nuclear run-on experiments. Several arguments suggest that any transcripts detected from R1 and R2 would represent cotranscription with the rRNA genes. First, transcripts initiating at sites within the insertions have not been observed by electron microscopy (10, 35). Second, we have been unable to detect promoters associated with the R2 elements (25). Finally, transcripts can be detected of 5′-truncated copies of the elements or of foreign sequences inserted into the R2 site (20).

In the run-on experiments, ^{32}P-labeled RNAs synthesized during a 15-min incubation of embryonic nuclei were hybridized to immobilized DNA fragments corresponding to different regions of the rDNA unit. Because no sequences are unique to uninserted units, the level of transcription from the R1 and R2 insertions was compared to the total transcription of all units. Three regions of the rDNA repeat were tested to represent the combined transcription of all units: the ETS region, the ITS1 region, and a segment of the 28S gene upstream of the R1 and R2 insertion sites. The ITS1 and 28S

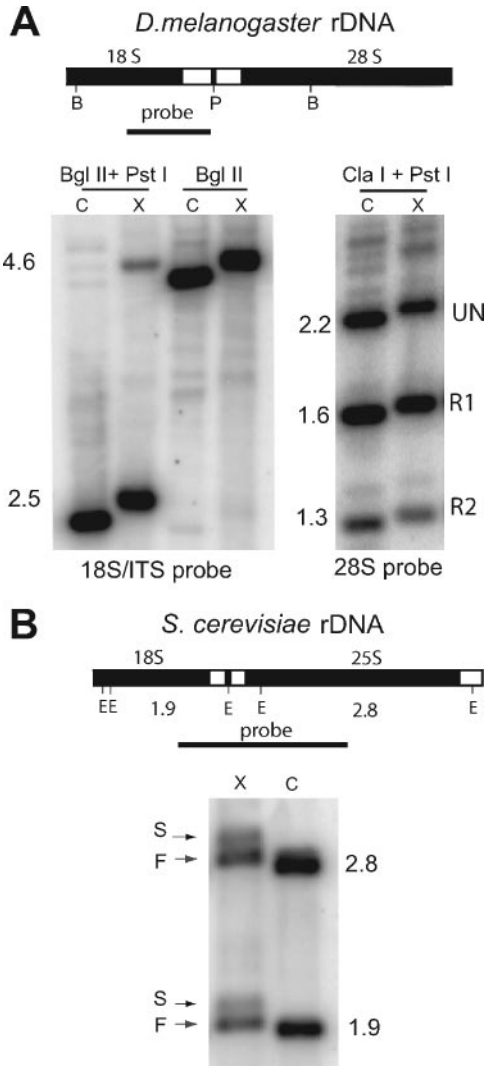

FIG. 5. Comparison of psoralen cross-linking of the rDNA units in *D. melanogaster* and *S. cerevisiae.* (A) Aliquots of isolated 1- to 16-h embryonic nuclei from *D. melanogaster* were either cross-linked with psoralen or with a buffer control. Left panel: after cross-linking, the DNA was extracted, digested with BglII or BglII plus PstI, blotted, and probed with a fragment from the 18S gene and ITS1 sequences. Show above the gels is a diagram of a segment of the rDNA locus indicating the locations of the BglII and PstI sites and the probe used. Cross-linked DNA corresponds to lanes labeled X, and uncross-linked lanes are labeled C. Right panel: after cross-linking and extraction, the DNA was digested with ClaI and PstI and hybridized with a 28S probe adjacent to the downstream ClaI site (see diagram in Fig. 3A). (B) Psoralen cross-linking of *S. cerevisiae* rDNA units. The cross-linking conditions were the same as those described previously (15). The diagram at the top shows the location of the EcoRI restriction sites as well as the hybridization probe used. The separation of units into the inactive fast (F) and active slow (S) fractions can be seen for both the 1.9-kb 18S gene and the 2.8-kb 25S gene fragments.

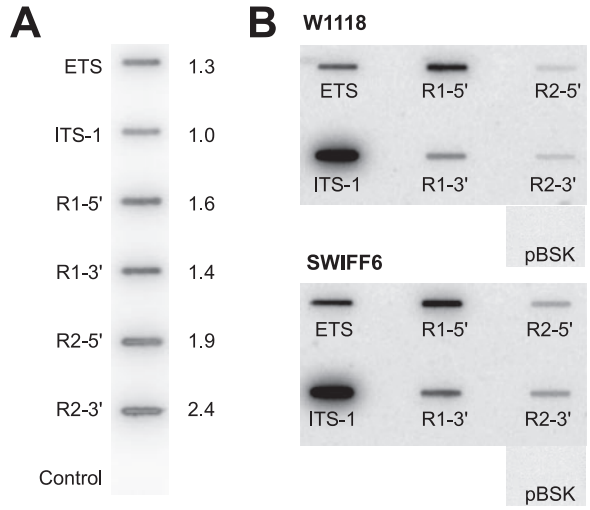

FIG. 6. Nuclear run-on transcription assays of inserted and uninserted rDNA units. (A) Determination of the relative hybridization efficiencies of the various DNA fragments. See Materials and Methods for experimental details. An unrelated mouse interferon DNA fragment (control) was included in the hybridization as a negative control. The numbers are averaged from two experiments, with the efficiency of the ITS-1 DNA set at 1. (B) One example of the nuclear run-on hybridization (experiment A in Table 2). DNA fragments (500 bp) corresponding to the regions of the inserted and uninserted rDNA units as shown in Fig. 1 were bound to nitrocellulose paper (31) and hybridized with RNA synthesized in 3- to 20-h embryo nuclei during a 15-min incubation at 37°C. Equal amounts of pBluescript II SK vector DNA (pBSK) were bound to the membrane as a negative control.

levels were similar in the various trial experiments, while the ETS hybridizations were more variable and averaged eightfold lower than the ITS1 or 28S hybridizations (see examples in Fig. 6). Presumably the ETS sequences near the promoter of the rDNA unit are either more rapidly degraded or become depleted of polymerase. We used RNA hybridization to the ITS1 region of the rDNA as the transcription standard in the following experiments, because the low levels of R1 and R2 transcripts detected on Northern blots or by RNA protection assays (20, 37, 43) suggest that if the inserts are transcribed, their RNA is quickly degraded like that of the ITS.

Nuclear run-on transcription assays were carried out with two lines: w^{1118}, the line used for the previous chromatin accessibility studies in this report, and SWIFF6, a line derived from w^{1118} but containing somewhat higher levels of R1 and R2 insertions (20). Because we needed to compare hybridization signals to different DNA fragments, the relative hybridization efficiency of each fragment was determined (Fig. 6A; also see Materials and Methods). The hybridization results from one nuclear run-on experiment are shown in Fig. 6B, while Table 2 summarizes four different experiments. All values in Table 2 were corrected for their hybridization efficiencies and were presented relative to the ITS1 signal, set at 100 in each experiment.

Several parameters were tested in the run-on assays. To provide additional evidence that the R1 and R2 transcripts were derived from cotranscription by RNA polymerase I rather than by RNA polymerase II, the nuclei were assayed in the presence (experiment B) or the absence (experiment A) of α-amanitin (42). The presence of α-amanitin had little effect on the relative transcript levels of R1 and R2, with transcription of the insertions in line SWIFF6 somewhat reduced in the presence of α-amanitin but somewhat increased in line w^{1118}. Reducing the size of the DNA fragments bound to the nitrocellulose filters to 250 bp instead of 500 bp (experiment C) and conducting the incubations at 25°C instead of 37°C (experiment D) also did not consistently change the levels of R1 and R2 transcripts compared to that of ITS1. Finally, we also conducted run-on assays with nuclei isolated from adult animals (data not shown). The levels of both R1 and R2 transcripts relative to that of ITS1 were two- to threefold higher in the adult female nuclei; however, the level of ITS1 transcripts was reduced 10- to 20-fold compared to that in embryonic nuclei, making quantitation less accurate. Based on the ITS1 signal, the total transcript levels of the rDNA units in adult male nuclei were even lower. Therefore, the following discussions are based on the transcription levels obtained from only the experiments conducted with embryonic nuclei.

As summarized in Table 2, the SWIFF6 line showed higher levels of R1 and R2 transcription than the w^{1118} line, consistent with the higher fraction of R1 and R2 insertions in SWIFF6 (20). Surprisingly, in both lines transcription of the 3′ end of the R1 element was about threefold lower than that of the 5′ end. This suggests that most transcription complexes fail to reach the 3′ end of the R1 element or that the 3′ RNAs are more rapidly degraded. In the case of R2, the levels of 5′ and 3′ end transcripts were more similar. However, nearly half of the R2 elements in these strains are 5′ truncated (i.e., do not contain the region being monitored by the 5′ probe) (20). If 5′-truncated insertions are cotranscribed at the same rate as full-length insertions, then many R2 transcription complexes also fail to reach the 3′ end of the element.

These run-on experiments indicated that the R1 and R2 elements were transcribed at measurable levels. To estimate how efficiently the inserted units were transcribed relative to transcription of the uninserted units, the transcription values of their 5′ ends were divided by the fraction of the rDNA units that contained each insertions. In the case of R1, 38% (w^{1118}) and 45% (SWIFF6) of the rDNA units contained full-length

TABLE 2. Nuclear run-on transcription assays to detect R1 and R2 transcripts

Expt	Line	ITS-1 signal	Relative hybridization signal[d]			
			R1 element		R2 element	
			5′	3′	5′	3′
A	SWIFF6	100	14.6	4.4	1.2	1.0
	w^{1118}	100	4.4	1.4	0.2	0.3
B[a]	SWIFF6	100	9.6	3.8	0.7	0.8
	w^{1118}	100	7.5	2.1	0.3	0.3
C[b]	SWIFF6	100	7.5	3.8	0.8	1.0
D[c]	w^{1118}	100	9.9	3.0	1.0	0.3
Avg	SWIFF6		10.6	4.0	0.9	0.9
	w^{1118}		7.3	2.2	0.5	0.3

[a] As experiment A, but conducted in 100 μg/ml α-amanitin.
[b] As experiment A, but the RNA was hybridized to 250-bp DNA fragments instead of 500 bp.
[c] As experiment A, but the run-on incubations were performed at 25°C instead of 37°C.
[d] Hybridization signal relative to the signal for ITS-1.

insertions. Thus, full-length R1-inserted units appear to be transcribed at 17% and 24%, or about one fifth, of the efficiency of the uninserted units. In the case of R2, 7.5% (w^{1118}) and 9.5% (SWIFF6) of the rDNA units contained full-length insertions. Thus, full-length R2-inserted units appear to be transcribed at 7% and 11%, or about 1/10 the efficiency of the uninserted units.

DISCUSSION

The 28S rRNA genes of *D. melanogaster* were among the first genes to be described that contained disruptions in their coding regions (28, 63). Initial studies attempted to determine whether these insertions were similar to the introns in protein coding genes and destined to be spliced from a primary RNA transcript. Northern blots of nuclear RNA revealed only low levels of cotranscripts from either insertion, arguing against their functioning as introns (37, 43). More dramatic were the electron microscopic observations of active rDNA units using the "Miller spreading technique" (51). Chooi and Laird observed rDNA transcription units variably longer than expected for uninserted units, but few extended to a length consistent with that of a full-length R1 or R2 cotranscript with the rRNA genes (10, 41). Jamrich and Miller suggested that less than 1% of the transcription complexes they observed in *D. melanogaster* were of a length consistent with the cotranscription of full-length insertions (35). These studies, combined with the discovery that small deletions of the 28S genes were often found at the 5′ junctions of the insertions (17, 57), provided convincing evidence that the inserted units were not being used to produce functional 28S rRNA, thus ending most investigations into the R1 and R2 insertions.

The discovery that R1 and R2 insertions in the rDNA units are active retrotransposable elements renewed interest in their expression, because transcription even at low levels could be a critical component of their regulation. The run-on transcription assays reported here indicated that R1- and R2-inserted rDNA units of *D. melanogaster* are transcribed at significant levels. Once activated, the rates of transcription initiation of inserted and uninserted units are similar, because the same densities of RNA polymerase complexes were observed by microscopy for the rare long (inserted) transcripts as for the shorter (uninserted) transcripts (10). Correcting for the fraction of the rDNA locus containing the insertions, full-length R1-inserted units are activated at about 1/5 and full-length R2-inserted units at about 1/10 of the rate of activation of the uninserted units. Inserted rDNA units may be activated at even higher relative levels if the R1 or R2 transcripts are more rapidly degraded than the ITS1 transcript used for comparison.

What explains the low levels of "long transcripts" in the electron microscopic studies? Our run-on data also suggest that most of the transcription complexes on the inserted units do not extend to the 3′ end of the insertion elements. Run-on transcripts from the 5′ end of the R1 elements were threefold more abundant than transcripts from the 3′ end (Table 2). Because 20% of the R1 insertions in the *Drosophila* strains used in the assays are 5′ truncated, containing 1 kb or less of the 3′ end of the element, cotranscription of these 5′-truncated R1 elements could account for most of the 3′ transcripts detected in the run-on assays. In a similar manner, while the levels of R2 5′ and 3′ run-on transcripts were about equal, half of the R2 insertions in the assayed lines do not contain the 5′ end of the element, again suggesting that transcription of the 5′-truncated copies could account for most of the 3′ R2 transcripts detected.

As additional support for premature terminations within inserted units, histone H3.3, which is associated with transcription (3), has been shown to accumulate in the first 1 kb of the R1 sequences but not in regions further downstream (S. Henikoff, personal communication). If most of the transcription complexes on the inserted units do not extend to the end of the insertion elements, our run-on results do not contradict the earlier electron microscopic studies of rRNA transcription. Transcription complexes that terminate within the first 1 to 2 kb of the R1 and R2 insertions would appear in electron microscopic observations to be of similar length to the complexes derived from uninserted units (the R1 and R2 insertion sites are 1.3 kb from the normal termination site of the rDNA unit; see Fig. 1). Thus, scoring by electron microscopy only transcription complex length significantly underestimated the level of transcription associated with inserted rDNA units. Indeed, direct electron microscopic evidence that transcription complexes were terminating within the insertion elements was obtained in the early studies. Chooi (10) observed two classes of intergenic spacer lengths between consecutive rDNA transcription units of *D. melanogaster*: a shorter class consistent with the 3- to 5-kb physical lengths of the intergenic spacer and a longer class consistent with transcription truncations within the R1 or R2 elements.

The only apparent conflict that exists between our run-on data and the earlier reports of R1 and R2 transcript levels is that full-length transcripts of R2 elements are more readily detected on Northern blots than full-length R1 transcripts (37, 43), while the run-on transcription data suggested that the R1-inserted units are more frequently activated for transcription than the R2-inserted units. We suggest this difference between the number of units activated for transcription and actual transcript levels is a result of the more-efficient termination of transcription within R1 elements or the greater stability of R2 transcripts.

What fractions of the rDNA units are transcribed? The number is likely to vary among tissues and strains, but our nuclease digestion and histone modification assays suggest that these fractions are quite low (Fig. 2 to 4). The most sensitive assay for detecting the level of active rDNA units is psoralen cross-linking (13, 15, 16, 47). However, our psoralen cross-linking study did not reveal active units, suggesting such units represent less than 10% of the units in any of the bands we detected on Southern blots (Fig. 5). Based on the run-on data, the highest proportion of active units would be the uninserted units. The w^{1118} line used for this study contained 300 rDNA units on each of the X and Y chromosomes, 40% of which are uninserted (i.e., about 240 uninserted units per cell) (20, 34). If less than 10% of the uninserted units are active, then the absolute number of such units is less than 24. This surprisingly low number is in fact consistent with that estimated from other studies. Only an estimated 50 rDNA units (both inserted and uninserted) are sufficient to avoid the *bobbed* phenotype (20, 22, 30). Electron microscopic observations of early embryo development suggested an average of 30 transcriptionally ac-

tive rDNA units in each nucleolus of *D. melanogaster* (50). The requirement for such low numbers of functional rDNA units in *Drosophila* could explain why more than 75% of the units can be inserted in strains of *D. melanogaster* (34) and why more than 90% of the rDNA units can be inserted in strains from other *Drosophila* species (32, 44, 49).

How are the active units arranged within the nucleolus? Electron microscopic observations suggest that transcriptionally active rDNA units are frequently consecutive, suggesting that in any cell, transcriptional activity is clustered within one or a few regions of the rDNA loci (10, 11, 26, 35). A recent report on the replacement of histone H3.3 in actively transcribed genes also suggested that transcriptionally active and inactive rDNA units are physically separated (3). Because inserted and uninserted rDNA units are extensively interspersed across the rDNA locus in *D. melanogaster* (10, 63; W. Burke, K. Averbeck, and T. Eickbush, unpublished data), a simple model to explain our R1 and R2 transcription data is that within these "activated domains" all rDNA units are transcriptionally active independently of whether they are inserted or uninserted. The lower levels of transcription of the R1- and R2-inserted units would simply reflect the fraction of these units in the active domains and the advantage to the cells of activating those regions of the locus with the highest fraction of uninserted units. In this model, most control over R1 and R2 activity would occur after transcription initiation. We have recently detected isofemale lines of *Drosophila simulans* with active R2 retrotransposition (66). This R2 activity is correlated with high levels of stable full-length R2 transcripts on Northern blots (D. G. Eickbush, X. Zhang, J. Ye, and T. H. Eickbush, unpublished data). We should be able to determine if this R2 retrotransposition activity is associated with increased rates of R2 transcription, a reduction in the rate of termination within the element, or an increase in the stability of the R2 transcripts.

R1 and R2 have been stably associated (vertically transmitted) with insect lineages since the origin of arthropods (6, 23, 48). The competition between the drive of these elements to survive by increasing their numbers and that of the hosts to bring about their elimination is but one of the many battlefields in the genomic war that is being fought in all organisms. Just as this war between the genome and mobile elements has been postulated to give rise to new types of gene regulation (e.g., DNA methylation and RNA interference) (55, 65), it can be postulated that the battle between R1 and R2 and the rDNA locus has given rise to new means of regulating the expression of the rDNA units. These new mechanisms could involve the activation of only a small number of genes, mechanisms to promote termination of the transcription apparatus within the insertions, or new means to process the rRNA transcripts.

ACKNOWLEDGMENTS

We thank Tom Maniatis for allowing some of these experiments to be conducted in his laboratory. We also thank Marty Gorovsky for useful discussion and Xin Bi and Danna Eickbush for comments on the manuscript.

This work was supported by National Institutes of Health grant GM42790 to T.H.E.

REFERENCES

1. **Abad, J. P., M. Carmena, S. Baars, R. D. Saunders, D. M. Clover, P. Ludena, C. Sentis, C. Tyler-Smith, and A. Villasante.** 1992. Dodeca satellite: a conserved G+C-rich satellite from the centromeric heterochromatin of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA **89:**4663–4667.
2. **Anzai, T., M. Osanai, M. Hamada, and H. Fujiwara.** 2005. Functional roles of 3′-terminal structures of template RNA during in vivo retrotransposition of non-LTR retrotransposon, R1Bm. Nucleic Acids Res. **33:**1993–2002.
3. **Ahmad, K., and S. Henikoff.** 2002. The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. Mol. Cell **9:**1191–1200.
4. **Averbeck, K. T., and T. H. Eickbush.** 2005. Monitoring the mode and tempo of concerted evolution in the Drosophila melanogaster rDNA locus. Genetics **171:**1837–1846.
5. **Bi, X., M. Braunstein, G. Shei, and J. R. Broach.** 1999. The yeast *HML* I silencer defines a heterochromatin domain boundary by directional establishment of silencing. Proc. Natl. Acad. Sci. USA **96:**11934–11939.
6. **Burke, W. D., H. S. Malik, W. C. Lathe III, and T. H. Eickbush.** 1998. Are retrotransposons long-term hitchhikers? Nature **392:**141–142.
7. **Burke, W. D., F. Muller, and T. H. Eickbush.** 1995. R4, a non-LTR retrotransposon specific to the large subunit rRNA genes of nematodes. Nucleic Acids Res. **23:**4628–4634.
8. **Burke, W. D., D. Singh, and T. H. Eickbush.** 2003. R5 retrotransposons insert into a family of infrequently transcribed 28S rRNA genes of Planaria. Mol. Biol. Evol. **20:**1260–1270.
9. **Cavalli, G., V. Orlando, and R. Paro.** 1999. Mapping DNA target sites of chromatin-associated proteins by formaldehyde cross-linking in *Drosophila* embryos, p. 20–37. *In* W. A. Bickmore (ed.), Chromosome structural analysis: a practical approach. Oxford University Press, Oxford, United Kingdom.
10. **Chooi, W. Y.** 1979. The occurrence of long transcription units among the X and Y ribosomal genes of *Drosophila melanogaster*: transcription of insertion sequences. Chromosoma **74:**57–74.
11. **Chooi, W. Y., and K. R. Lieby.** 1984. Electron microscopic evidence for RNA polymerase loading at repeated sequences in non-transcribed spacers in *D. virilis*. Exp. Cell Res. **154:**181–190.
12. **Christensen, S. M., and T. H. Eickbush.** 2005. R2 target-primed reverse transcription: ordered cleavage and polymerization steps by protein subunits asymmetrically bound to the target DNA. Mol. Cell. Biol. **25:**6617–6628.
13. **Conconi, A., J. M. Sogo, and C. A. Ryan.** 1992. Ribosomal gene clusters are uniquely proportioned between open and closed chromatin structures in both tomato leaf cells and exponentially growing suspension cultures. Proc. Natl. Acad. Sci. USA. **89:**5256–5260.
14. **Conconi, A., R. M. Widmer, T. Koller, and J. M. Sogo.** 1989. Two different chromatin structures coexist in ribosomal RNA genes throughout the cell cycle. Cell **57:**753–761.
15. **Dammann, R., R. Lucchini, T. Koller, and J. M. Sogo.** 1993. Chromatin structures and transcription of rDNA in yeast *Saccharomyces cerevisiae*. Nucleic Acids Res. **21:**2331–2338.
16. **Dammann, R., R. Lucchini, T. Koller, and J. M. Sogo.** 1995. Transcription in the yeast rRNA gene locus: distribution of the active gene copies and chromatin structure of their flanking regulatory sequences. Mol. Cell. Biol. **15:**5294–5303.
17. **Dawid, I. B., and M. L. Rebbert.** 1981. Nucleotide sequence at the boundaries between gene and insertion regions in the rDNA of D. melanogaster. Nucleic Acids Res. **9:**5011–5020.
18. **Dawid, I. B., and M. L. Rebbert.** 1986. Expression of ribosomal insertion in Drosophila: sensitivity to intercalating drugs. Nucleic Acids Res. **14:**1267–1277.
19. **Dekker, C. A.** 1960. Nucleic acids. Selected topics related to their enzymology and chemistry. Annu. Rev. Biochem. **29:**453–474.
20. **Eickbush, D. G., and T. H. Eickbush.** 2003. Transcription of endogenous and exogenous R2 elements in the rDNA gene locus of *Drosophila melanogaster*. Mol. Cell. Biol. **23:**3825–3836.
21. **Eickbush, T. H.** 2002. R2 and related site-specific non-LTR retrotransposons, p. 813–833. *In* N. L. Craig, R. Craigie, M. Gellert, and A. M. Lambowitz (ed.), Mobile DNA II. American Society of Microbiology, Washington, D.C.
22. **Franz, G., and W. Kunz.** 1981. Intervening sequences in ribosomal RNA genes and bobbed phenotype in *Drosophila hydei*. Nature **292:**638–640.
23. **Gentile, K. L., W. D. Burke, and T. H. Eickbush.** 2001. Multiple lineages of R1 retrotransposable elements can co-exist in the rDNA locus of Drosophila. Mol. Biol. Evol. **18:**235–245.
24. **George, J. A., W. D. Burke, and T. H. Eickbush.** 1996. Analysis of the 5′ junctions of R2 insertions with the 28S gene: implications for non-LTR retrotransposition. Genetics **142:**853–863.
25. **George, J. A., and T. H. Eickbush.** 1999. Conserved features at the 5′ end of Drosophila R2 retrotransposable elements: implications for transcription and translation. Insect Mol. Biol. **8:**3–10.
26. **Glatzer, K. H.** 1979. Lengths of transcribed rDNA repeating types in spermatocytes of *Drosophila hydei*: only genes without an intervening sequence are expressed. Chromosoma **75:**161–175.
27. **Glover, D. M.** 1981. The rDNA of *Drosophila melanogaster*. Cell **26:**297–298.
28. **Glover, D. M., and D. S. Hogness.** 1977. A novel arrangement of the 18S and

28S sequences in a repeating unit of Drosophila melanogaster. Cell **10:**167–176.
29. **Grummt, I., and C. S. Pikaard.** 2003. Epigenetic silencing of RNA polymerase I transcription. Nat. Rev. Mol. Cell Biol. **4:**641–649.
30. **Hawley, R. S., and C. H. Marcus.** 1989. Recombinational controls of rDNA redundancy in Drosophila. Annu. Rev. Genet. **23:**87–120.
31. **Hirayoshi, K., and J. T. Lis.** 1999. Nuclear run-on assays: assessing transcription by measuring density of engaged RNA polymerases. Methods Enzymol. **304:**351–362.
32. **Hollocher, H., and A. R. Templeton.** 1994. The molecular through ecological genetics of abnormal abdomen in *Drosophila mercatorum.* VI. The nonneutrality of the Y chromosome rDNA polymorphism. Genetics **136:**1373–1384.
33. **Jakubczak, J. L., Y. Xiong, and T. H. Eickbush.** 1990. Type I (R1) and type II (R2) ribosomal DNA insertions of *Drosophila melanogaster* are retrotransposable elements closely related to those of *Bombyx mori*. J. Mol. Biol. **212:**37–52.
34. **Jakubczak, J. L., M. K. Zenni, R. C. Woodruff, and T. H. Eickbush.** 1992. Turnover of R1 (type I) and R2 (type II) retrotransposable elements in the ribosomal DNA of *Drosophila melanogaster*. Genetics **131:**129–142.
35. **Jamrich, M., and O. L. Miller.** 1984. The rare transcripts of interrupted rDNA genes in Drosophila melanogaster are processed or degraded during synthesis. EMBO J. **3:**1541–1545.
36. **Jenuwein, T., and C. D. Allis.** 2001. Translating the histone code. Science **293:**1074–1080.
37. **Kidd, S. J., and D. M. Glover.** 1981. *Drosophila melanogaster* ribosomal DNA containing type II insertions is variably transcribed in different strains and tissues. J. Mol. Biol. **151:**645–662.
38. **Kohorn, B. D., and P. M. M. Rae.** 1982. Accurate transcription of truncated ribosomal DNA templates in a Drosophila cell-free system. Proc. Natl. Acad. Sci. USA **79:**1501–1505.
39. **Kojima, K. K., and H. Fujiwara.** 2005. Long-term inheritance of the 28S rDNA-specific retrotransposon R2. Mol. Biol. Evol. **22:**2157–2165.
40. **Kolchinskii, A. M., R. P. Bashakidze, O. V. Preobrazhenskaya, V. L. Karpov, and A. D. Mirzabekov.** 1984. Chromatin structure of ribosomal genes of *Drosophila melanogaster.* Random arrangement of nucleosomes on DNA and peculiarities in the organization of the nontranscribed spacer. Mol. Biol. (Moscow) **18:**1141–1150.
41. **Laird, C. D., and W. Y. Chooi.** 1976. Morphology of transcription units in *Drosophila melanogaster*. Chromosoma **58:**193–218.
42. **Lindell, T. J., F. Weinberg, P. W. Morris, R. G. Roeder, and W. J. Rutter.** 1970. Specific inhibition of nuclear RNA polymerase II by alpha-amanitin. Science **170:**447–449.
43. **Long, E. O., and I. B. Dawid.** 1979. Expression of ribosomal DNA insertions in *Drosophila melanogaster*. Cell **18:**1185–1196.
44. **Long, E. O., and I. B. Dawid.** 1982. Repeated genes in eukaryotes. Annu. Rev. Biochem. **49:**727–764.
45. **Long, E. O., M. L. Rebbert, and I. B. Dawid.** 1981. Nucleotide sequence of the initiation site for rRNA transcription in Drosophila melanogaster: comparison of genes with and without insertions. Proc. Natl. Acad. Sci. USA **78:**1513–1517.
46. **Luan, D. D., M. H. Korman, J. L. Jakubczak, and T. H. Eickbush.** 1993. Reverse transcription of R2Bm RNA is primed by a nick at the chromosomal target site: a mechanism for non-LTR retrotransposition. Cell **72:**595–605.
47. **Luchini, R., and J. M. Sogo.** 1992. Different chromatin structures along the spacers flanking active and inactive *Xenopus* rRNA genes. Mol. Cell. Biol. **12:**4288–4296.
48. **Malik, H. S., W. D. Burke, and T. H. Eickbush.** 1999. The age and evolution of non-LTR retrotransposable elements. Mol. Biol. Evol. **16:**793–805.
49. **Malik, H. S., and T. H. Eickbush.** 1999. Retrotransposable elements R1 and R2 in the rDNA units of *D. mercatorum*: abnormal abdomen revisited. Genetics **151:**653–665.
50. **McKnight, S. L., and O. L. Miller.** 1976. Ultrastructural patterns of RNA synthesis during early embryogenesis of *Drosophila melanogaster*. Cell **8:**305–319.
51. **Miller, O. L., Jr., and B. R. Beatty.** 1969. Visualization of nucleolar genes. Science **164:**955–957.
52. **Mutskov, V. J., V. R. Russanova, S. I. Dimitrov, and I. G. Pashev.** 1996. Histones associated with non-nucleosomal rat ribosomal genes are acetylated while those bound to nucleosome-organized gene copies are not. J. Biol. Chem. **271:**11852–11857.
53. **Perez-Gonzalez, C. E., and T. H. Eickbush.** 2001. Dynamics of R1 and R2 elements in the rDNA locus of *Drosophila simulans*. Genetics **158:**1557–1567.
54. **Perez-Gonzalez, C. E., and T. H. Eickbush.** 2002. Rates of R1 and R2 retrotransposition and elimination from the rDNA locus of *Drosophila melanogaster*. Genetics **162:**799–811.
55. **Plasterk, R. H., and R. F. Ketting.** 2000. The silence of the genes. Curr. Opin. Genet. Dev. **10:**562–567.
56. **Roberts, W., C. Dekker, G. Rushisky, and C. Knight.** 1962. Studies on the mechanism of action of micrococcal nuclease. I. Degradation of thymus deoxyribonucleic acid. Biochim. Biophys. Acta **55:**664–673.
57. **Roiha, H., J. R. Miller, L. C. Woods, and D. M. Glover.** 1981. Arrangements and rearrangements of sequences flanking the two types of rDNA insertion in *D. melanogaster*. Nature **290:**749–753.
58. **Sandmeier, J. J., S. French, Y. Osheim, W. L. Cheung, C. M. Gallo, A. L. Beyer, and J. S. Smith.** 2002. RPD3 is required for the inactivation of yeast ribosomal RNA genes in stationary phase. EMBO J. **21:**4959–4968.
59. **Santoro, R., J. Li, and I. Grummt.** 2002. The nucleolar remodeling complex NoRC mediates heterochromatin formation and silencing of ribosomal gene transcription. Nat. Genet. **32:**393–396.
60. **Tautz, D., and G. A. Dover.** 1986. Transcription of the tandem array of ribosomal DNA in *Drosophila melanogaster* does not terminate at any fixed-point. EMBO J. **5:**1267–1273.
61. **Udvardy, A., C. Louis, S. Han, and P. Schedl.** 1984. Ribosomal RNA genes of *Drosophila melanogaster* have a novel chromatin structure. J. Mol. Biol. **175:**113–130.
62. **Wayne, R. L., Z. D. Sharp, and J. D. Procunier.** 1985. Preferential DNase I sensitivity of insert-free ribosomal RNA repeats of *Drosophila melanogaster*. Nucleic Acids Res. **13:**2869–2879.
63. **Wellauer, P. K., and I. B. Dawid.** 1977. The structural organization of ribosomal DNA in *Drosophila melanogaster*. Cell **10:**193–212.
64. **Wu, C.** 1989. Analysis of hypersensitive sites in chromatin. Methods Enzymol. **170:**269–289.
65. **Yoder, J. A., C. P. Walsh, and T. H. Bestor.** 1997. Cytosine methylation and the ecology of intergenomic parasites. Trends Genet. **13:**335–340.
66. **Zhang, X., and T. H. Eickbush.** 2005. Characterization of active R2 retrotransposition in the rDNA locus of *Drosophila melanogaster*. Genetics **170:**195–205.